Supporting Information

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SI Materials and Methods

Mice and Cell Lines. All mice were housed pathogen-free. GFP-LC3$^+$ mice were kindly provided by Noboru Mizushima at Tokyo Medical and Dental University, Tokyo. ULK1$^{-/-}$ mice were kindly provided by Mondira Kundu (St. Jude Children's Research Hospital, Memphis, TN) and were bred to GFP-LC3$^+$ mice to generate ULK1$^{-/-}$ GFP-LC3$^+$ mice. To generate mice with ATG7$^{-/-}$ macrophages, male ATG7$^{flox/flox}$ mice (kindly provided by Masaaki Komatsu at Tokyo Metropolitan Institute of Medical Science, Tokyo) were bred to female LysM-Cre$^+$ mice (kindly provided by Peter Murray, St. Jude Children's Research Hospital, Memphis, TN). These resulting mice were then bred with GFP-LC3 mice to generate LysM-Cre$^+$ or LysM-Cre$^-$ ATG7$^{flox/flox}$ GFP-LC3$^+$ mice. TIM4$^{-/-}$ mice were kindly provided by Vijay Kuchroo (Harvard University, Cambridge, MA) and were bred to GFP-LC3$^+$ mice to generate TIM4$^{-/-}$ GFP-LC3$^+$ mice.

Mouse bone marrow-derived macrophages were generated from bone marrow progenitors obtained from littermates. Freshly prepared bone marrow cells were cultured in RPMI medium 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 10 mM Hepes buffer, 50 μg/mL penicillin, and nonessential amino acids in the presence of 20 ng/mL recombinant mouse macrophage colony stimulating factor (Peprotech). Unattached cells were removed at days 3 and 6.

Wild-type murine embryonic fibroblasts (MEFs) were generated from day 13.5 embryos, as described (1). MEFs stably expressing mCerulean-Spectrin were generated by transfection of the linearized construct with Lipofectamine 2000 (Invitrogen) and subsequent kanamycin selection and cell sorting over a 2-wk period.

RAW-GFP-LC3 cells were grown in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μg/mL penicillin, nonessential amino acids, sodium pyruvate, and 2-mercaptoethanol (55 μM). RAW-GFP-LC3-TIM4-HA cells were generated by transfection of the linearized TIM4-HA construct with Lipofectamine 2000 and subsequent antibiotic selection, as described (2).

RIPK3$^{+/+}$ and RIPK3$^{-/-}$ SVEC cells were generated as previously described (3) and were grown in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 50 μg/mL penicillin.

Constructs. GFP-LC3 construct was kindly provided by Annete Khaled (University of Central Florida, Orlando, FL). mCerulean-Spectrin construct was generated by cloning spectrin cDNA into mCerulean-C1 (a gift from D. Piston, Vanderbilt University, Nashville, TN) downstream of mCerulean using EcoRI and SalI restriction sites. TIM4-HA and Ø;-HA (empty vector) constructs were kindly provided by Vijay Kuchroo (Harvard University, Cambridge, MA), as previously described (2).

Induction of Death. Apoptosis was induced in RIPK3$^{-/-}$ SVEC cells by treatment with TNFα (20 ng/mL) and cyclohexamide (CHX, 2 μg/mL) for 8 h (3) or UV irradiation (20 J/m^2). Wild-type SVEC cells were induced to undergo necrosis by repeated freeze/thaw cycles (3×). To induce RIPK3-dependent necrosis, wild-type SVEC cells were treated with TNFα (20 ng/mL) and the pan-caspase inhibitor zVAD-fmk (50 μM) for 8 h (3).

Flow Cytometry Analysis. To assess cell death, cells were stained with propidium iodide and Annexin-V as previously described (3). Bone marrow-derived macrophage cultures were stained with CD11b-APC (M1/70) and F4/80-PE (BM8), both from eBioscience. Anti-TIM4 (3A1, 5G3, and 3H11) was kindly provided by Vijay Kuchroo (Harvard University, Cambridge, MA), and detection was achieved by secondary labeling with goat anti-rat IgG (heavy and light chain)–Alexa Flour 647 (Invitrogen).

Staining of Dead Cells. For visualization of dead cells, unattached cells were collected and washed twice with PBS. Dead cells were resuspended at 10^7 cells/mL in PBS and were labeled with 5 μM SytoRed (Invitrogen) for 15 min at room temperature per manufacturer's instructions. Labeled dead cells were washed twice with PBS and used in the phagocytosis assay at a ratio of 10:1 (dead cell:macrophage).

Autophagy and LAP Induction. Macrophages cultures were induced to undergo autophagy by 24 h of rapamycin treatment (200 nM). Alexa Fluor 594-labeled zymosan particles (Invitrogen) or unconjugated latex microspheres (3 μm; Polysciences) were added to macrophage cultures at a ratio of 8:1 (particle:cell). Class III PI3K activity was inhibited by 3-MA treatment; apoptotic cells were added to the macrophages cultures and allowed to become engulfed. Before translocation of GFP-LC3 to the dead cell-containing phagosome, 3-MA (1 mM) was added, and the capture of time-lapse images from the same field was resumed. To label lysosomal compartments, macrophages were preloaded with Lysotracker red, according to the manufacturer's instructions (Invitrogen).

siRNA Gene Silencing of ATG5 and Beclin1. Knockdown of mouse ATG5 using siRNA was performed as previously described (4). Knockdown of mouse Beclin1 was achieved by ON-TARGET plus siRNA obtained from Dharmacon. Primary macrophages were transfected with 50 μM of each siRNA with Lipofectamine RNAi Max according to the manufacturer's recommendations (Invitrogen).

Real-Time RT-PCR. Total RNA was isolated from macrophage cells 24 h post transfection using TRIzol (Gibco BRL) according to the manufacturer's instructions. First-strand synthesis was performed using M-MLV reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR GREEN PCR master mix (Applied Biosystems) in an Applied Biosystems 7900HT thermocycler using SyBr Green detection protocol as outlined by the manufacturer using the following PCR conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. mRNA expression was normalized against L32, allowing comparison of mRNA levels. The following primers were used: mouse ATG5 (forward primer: 5-AACTGAAAGAGAAGCAGAACCA-3; reverse primer: 5-TGTCTCATAACCTTCTGAAAGTGC-3), mouse Beclin1 (forward primer: 5-GGCCAATAAGATGGGTCTGA-3; reverse primer: 5-GCTGCACACAGTCCAGAAAA-3), and mouse L32 (forward primer: 5-GAAACTGGCGGAAACCCAX-3; reverse primer: 5-GGATCTGGCCCTTGAACCTT-3).

Cell Lysis and Immunoblotting. Cells were lysed in RIPA buffer for 30 min on ice [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate (DOC), 0.1% SDS, protease inhibitor tablet (Roche), 1 mM NaF, 1 mM Na_3VO_4, and 1 mM PMSF]. After centrifugation (1,770 × g for 15 min at 4 °C), supernatants were analyzed by SDS/PAGE. Anti-LC3B antibody was from Cell Signaling. Anti-actin antibody (C4) was from MP Biomedicals. Anti-ATG7 antibody was from Cell Signaling.

Time-Lapse Imaging and Microscopy. Confocal microscopy was performed using the following systems described below.

Spinning disk confocal microscopy. Spinning disk confocal microscopy (SDC) on live cells was performed with a Marianas SDC imaging system (Intelligent Imaging Innovations, Inc., Denver, CO) consisting of a CSU22 confocal head (Yokogowa Electric), DPSS lasers (CrystaLaser) with wavelengths of 445, 473, 523, 561, and 658 nm, and a Carl Zeiss 200M motorized inverted microscope (Carl Zeiss MicroImaging), equipped with spherical aberration correction optics. Temperature was maintained at ~37 °C and 5% CO_2 using an environmental control chamber (Solent Scientific). Images were acquired with a Zeiss Plan-Neofluar 40× 1.3 N.A. differential interference contrast (DIC) objective on a CascadeII 512 EMCCD (Photometrics), using SlideBook 4.2 software.

Laser scanning confocal microscopy. Laser scanning confocal microscopy (LSCM) on live cells was performed with a Nikon TE2000-E inverted microscope equipped with a C1Si confocal system (Nikon), an argon ion laser at 488 nm, and DPSS lasers at 404 and 561 nm (Melles Griot). Temperature was maintained at ~37 °C and 5% CO_2 using an environmental control chamber (InVivo Scientific). Images were taken at the intervals indicated in figure legends 1–4 and S1–S7 using an oil-immersion Nikon Plan Fluor 40× 1.3 N.A. objective with phase-contrast optics.

For time-lapse microscopic imaging of phagocytosis, macrophages were plated on fibronectin-coated glass bottom dishes (MatTek). GFP-LC3 translocation to the dead cell-containing phagosome was quantified by acquiring a time-lapse movie for 18 h and counting the number of GFP-LC3$^+$ dead cell-containing phagosomes out of the total number of engulfed dead cells for that period. For each condition, three independent experiments were performed, and the mean with SD error bars was represented. At the end of each experiment, we confirmed that particles were completely internalized by differential focusing.

Electron Microscopy. Macrophages grown on coverslips were prefixed in glutaraldehyde (2.5%) dissolved in 0.1 M Tousimis phosphate sodium buffer (pH 7.35) for 1 h at room temperature. The coverslips were then washed twice with 0.1 M sodium cacodylate buffer, treated with 1% tannic acid in 0.1 M sodium cacodylate buffer for 60 min, and then submerged in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 60 min. After dehydration in a graded ethanol series, the cells were cleared with propylene oxide for 10 min and dried overnight. The following day the samples were embedded, and pictures were taken with a JEOL 1200 EX-II transmission electron microscope with a digitalized Gatan camera (ES500W).

Quantification of Phagocytosis. Phagocytosis was calculated by dividing the number of macrophages that had fully engulfed a SytoRed-labeled apoptotic cell by the total number of macrophages in the field of vision at 60, 120, and 240 min after feeding. Calculation is an average of four independent time-lapse movies with more than 25 total macrophages counted per frame.

Inhibition of TIM4. Anti-TIM4 mAb 3H11 was previously described (2), and rIgG1 isotype control was from eBioscience. Twenty micrograms per milliliter of anti-TIM4 3H11 mAb or rIgG1 isotype control were added to macrophage cultures 1 h before the addition of dead cells.

Liposome Preparation. L-α-phosphatidylserine (PS; 870336C) and L-α-phophatidylcholine (PC; 840051C) were purchased from Avanti Polar Lipids. Liposomes were prepared as previously described (5). Briefly, liposomes were prepared from either 100% phosphatidylcholine (100%PC) or 70% phosphatidylcholine/30% phosphatidylserine (70% PC/30% PS) and labeled with 25 mg/mL Dextran–Texas Red (Invitrogen). Liposomes were added to macrophage cultures at a ratio of 10:1 (liposomes: macrophage). After 3 h of incubation, macrophages were washed gently with PBS to remove any nonengulfed liposomes.

Cytokine Detection. Supernatants were collected from macrophages fed with apoptotic, necrotic, or RIPK3-necrotic cells for 24 h. Cytokines (IL-1β, IL-6, IL-10, TGF-β) released into supernatant were analyzed by Luminex technologies (Millipore).

Nematode Strains and Reagents. Nematode strains were cultivated as described previously (6). Mutations used were LGI: opIs110 [Plim-7::yfp::act-5]. The integration site of opIs282 [Pced-1::yfp::rab-5, unc-119(+)] is not mapped.

RNA Interference. Feeding RNAi was performed as previously described (7). Plates containing nematode growth medium (NGM)–agarose, 200 μg/mL ampicillin, and 2 mM isopropyl β-D-1 thiogalactopyranoside (RNAi plates) were inoculated with 300 μL of appropriate bacterial cultures (transformed with constructs for generation of double-stranded RNA under the control of the T7 promoter) and incubated for 8–12 h before addition of worms. Worms were then stained with acridine orange and apoptotic germ cells scored under an M2Bio epifluorescence dissecting microscope (Zeiss). Positive candidates were retested, and apoptotic germ cells were scored directly by DIC microscopy.

Cell Corpse Assay. Worms were placed on 2% agarose pads in M9, anesthetized with levamisole (3–5 mM; Sigma), and mounted under a coverslip for observation using a Leica DM-RA microscope equipped with DIC (Normarski) optics and standard epifluorescence with filters appropriate for detection of YFP or GFP. Acridine orange (Molecular Probes) staining was performed as described previously (8).

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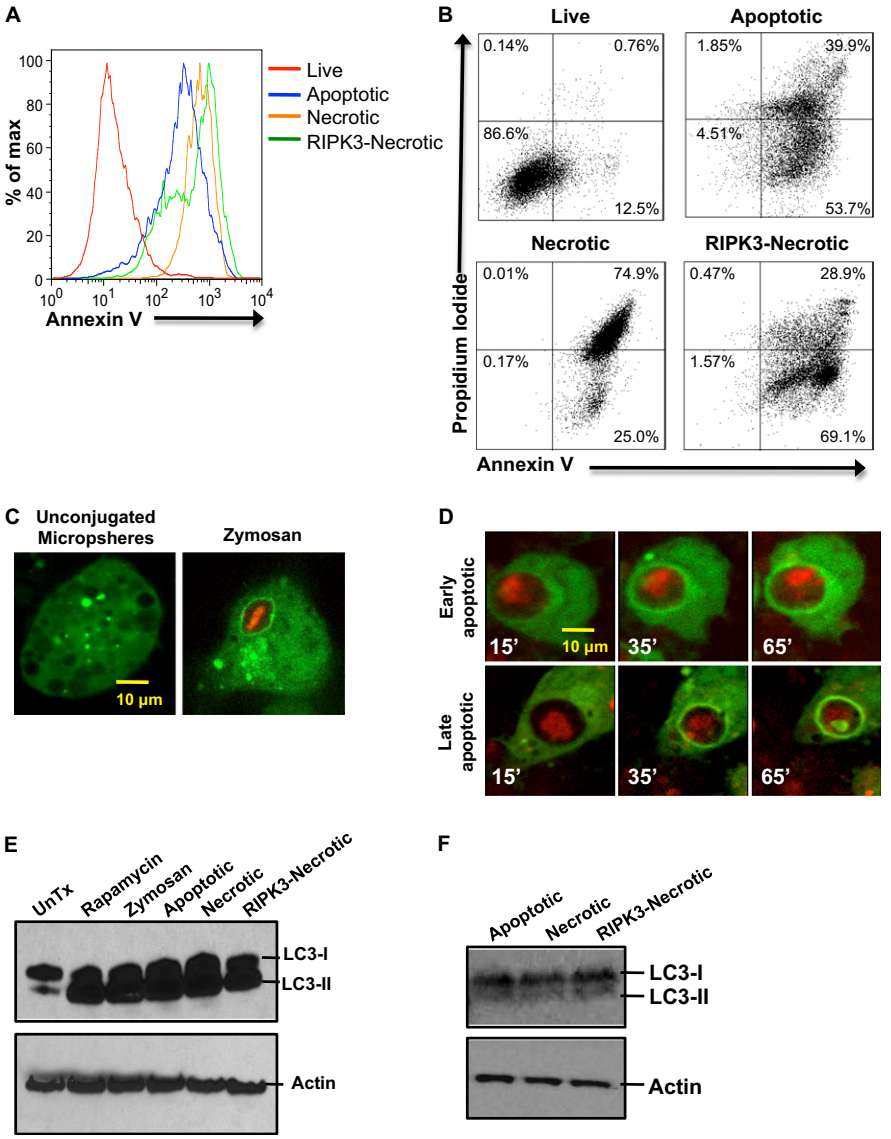

Fig. S1. Analysis of apoptotic, necrotic, and RIPK3-necrotic cells. (*A* and *B*) SVEC cells were induced to undergo apoptosis, necrosis, or RIPK3-necrosis, stained for propidium iodide and Annexin-V after 8 h of treatment, and analyzed by flow cytometry. (*C*) Macrophages were fed with unconjugated latex microspheres (3 μm) or Alexa Fluor-594 zymosan (red), and translocation of GFP-LC3 to the phagosome was followed by time-lapse video microscopy for 2 h (representative frames are shown; $n = 4$). (*D*) Macrophages were fed UV-induced early apoptotic or TNFα/CHX-treated late apoptotic cells (SytoRed), and translocation of GFP-LC3 to the dead cell-containing phagosome was followed by time-lapse video microscopy for 18 h (representative frames are shown; $n = 4$). Time post engulfment is noted in minutes. (*E*) Macrophages were fed with zymosan, apoptotic cells, necrotic cells, or RIPK3-necrotic cells for 2 h. Untreated and rapamycin-treated macrophages were obtained at 24 h. Cell lysates were prepared and the levels of LC3-I and the lipidated form LC3-II were determined by Western blot. As a loading control, the same membrane was restained for actin. (*F*) Apoptotic cells, necrotic cells, or RIPK3-necrotic cells were lysed, and the levels of LC3-I and the active conjugated form LC3-II were determined by Western blot. As a loading control, the same membrane was restained for actin.

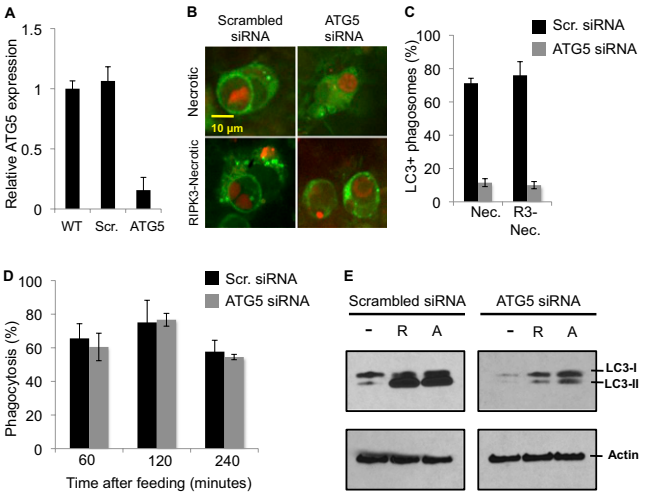

Fig. S2. Knockdown of ATG5 results in decreased translocation of GFP-LC3 to dead cell-containing phagosomes. (*A*) GFP-LC3^{+} macrophages were transfected with Scrambled (control) or ATG5 siRNA oligonucleotides. After 24 h, the level of ATG5 mRNA was quantified by real-time PCR. Error bars represent SD of three independent experiments. (*B*) GFP-LC3^{+} macrophages were transfected by Lipofectamine RNAi Max with Scrambled or ATG5 siRNA oligonucleotides. At 24 h after transfection, cells were fed necrotic or RIPK3-necrotic cells (SytoRed), and translocation of GFP-LC3 to the dead cell-containing phagosome was followed by time-lapse video microscopy for 3 h (representative frames are shown; $n = 4$). (*C*) The percentage of GFP-LC3^{+} dead cell-containing phagosomes ($n > 100$/group) was obtained from three independent time-lapse videos (3 h each) of macrophages transfected with Scrambled or ATG5 siRNA. Error bars represent SD of three independent experiments. (*D*) Quantification of phagocytosis as represented by the average percentage of phagocytosis at 60, 120, and 240 min post addition of apoptotic cells. Error bars represent SD of four independent experiments. (*E*) Macrophages transfected with Scrambled or ATG5 siRNA were fed with apoptotic cells ("A") for 2 h. Untreated (–) and rapamycin (R)-treated macrophages were obtained at 24 h. Cell lysates were prepared, and the levels of LC3-I and the active conjugated form LC3-II were determined by Western blot. As a loading control, the same membrane was restained for actin.

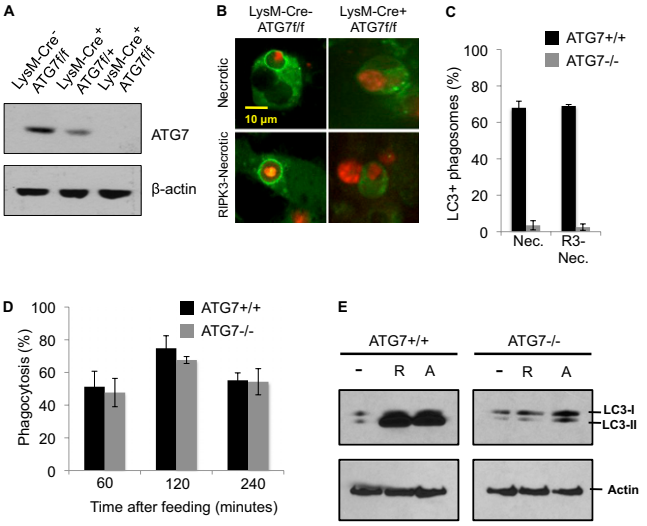

Fig. S3. Deletion of ATG7 in LysM-Cre^{+} ATG7$^{flox/flox}$ bone marrow-derived macrophages results in failure to translocate GFP-LC3 to the dead cell-containing phagosome. (*A*) Macrophages from LysM-Cre^{-} Atg7$^{f/f}$, LysM-Cre^{+} ATG7$^{f/+}$, and LysM-Cre^{+} ATG7$^{f/f}$ mice were lysed, and the levels of ATG7 were determined by Western blot. As a loading control, the same membrane was restained for actin. (*B*) LysM-Cre^{-} Atg7$^{f/f}$ GFP-LC3^{+} (ATG7$^{+/+}$) and LysM-Cre^{+} ATG7$^{f/f}$ GFP-LC3^{+} (ATG7$^{-/-}$) macrophages were fed necrotic or RIPK3-necrotic cells (SytoRed), and translocation of GFP-LC3 to the dead cell-containing phagosome was followed by time-lapse video microscopy for 3 h (representative frames are shown; $n = 4$). (*C*) The percentage of GFP-LC3^{+} dead cell-containing phagosomes ($n > 100$/group) was obtained from three independent time-lapse videos (3 h each) of ATG7$^{+/+}$ and ATG7$^{-/-}$ macrophages. Error bars represent SD of four independent experiments. (*D*) Quantification of phagocytosis as represented by the average percentage of phagocytosis at 60, 120, and 240 min post addition of apoptotic cells. Error bars represent SD of four independent experiments. (*E*) ATG7$^{+/+}$ and ATG7$^{-/-}$ macrophages were fed with apoptotic ("A") for 2 h. Untreated (–) and rapamycin (R)-treated macrophages were obtained at 24 h. Cell lysates were prepared, and the levels of LC3-I and the active conjugated form LC3-II were determined by Western blot. As a loading control, the same membrane was restained for actin.

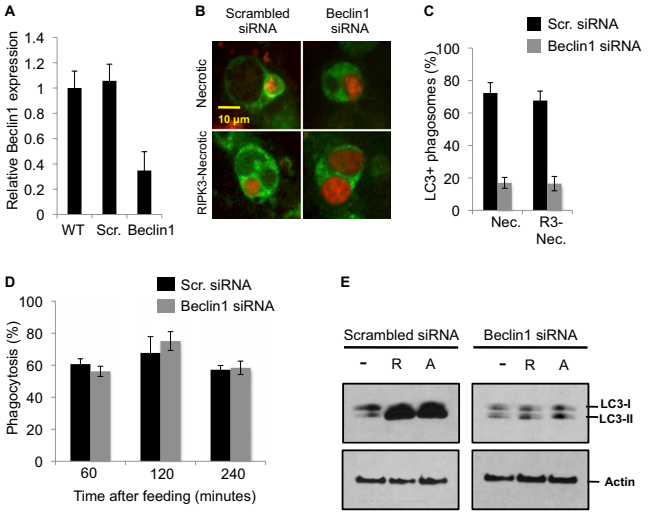

Fig. S4. Knockdown of Beclin1 results in failure to translocate GFP-LC3 to the dead cell-containing phagosome. (*A*) GFP-LC3$^+$ macrophages were transfected with Scrambled (control) or Beclin1 siRNA oligonucleotides. After 24 h, the level of Beclin1 mRNA was quantified by real-time PCR. Error bars represent SD of three independent experiments. (*B*) GFP-LC3$^+$ macrophages were transfected by Lipofectamine RNAi Max with Scrambled or Beclin1 siRNA oligonucleotides. At 24 h post transfection, cells were fed necrotic or RIPK3-necrotic cells (SytoRed), and translocation of GFP-LC3 to the dead cell-containing phagosome was followed by time-lapse video microscopy for 3 h (representative frames are shown; $n = 3$). (*C*) The percentage of GFP-LC3$^+$ dead cell-containing phagosomes ($n >$ 100/group) was obtained from three independent time-lapse videos (3 h each) of macrophages transfected with Scrambled or Beclin1 siRNA. Error bars represent SD of three independent experiments. (*D*) Quantification of phagocytosis as represented by average percentage of phagocytosis at 60, 120, and 240 min post addition of apoptotic cells. Error bars represent SD of four independent experiments. (*E*) Macrophages transfected with control or Beclin1 siRNA were fed with apoptotic cells ("A") for 2 h. Untreated (–) and rapamycin (R)-treated macrophages were obtained at 24 h. Cell lysates were prepared, and the levels of LC3-I and the active conjugated form LC3-II were determined by Western blot. As a loading control, the same membrane was restained for actin.

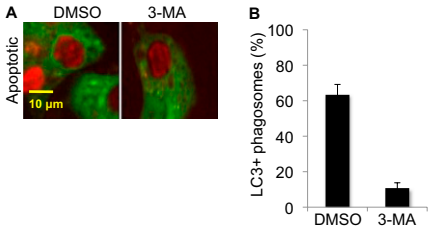

Fig. S5. Pharmacological inhibition of class III PI3K results in decreased LC3 translocation in response to the uptake of apoptotic cells. (*A*) GFP-LC3$^+$ macrophages were fed with apoptotic cells, and engulfment was allowed to occur. Before GFP-LC3 translocation to the apoptotic cell-containing phagosome, 3-MA (1 mM) was added, and the capture of time-lapse images from the same field was resumed (representative frames are shown; $n = 2$). (*B*) The percentage of GFP-LC3$^+$ dead cell-containing phagosomes ($n >$ 100/group) was obtained from three independent time-lapse videos (3 h each) of macrophages transfected with Scrambled or Beclin1 siRNA. Error bars represent SD of two independent experiments.

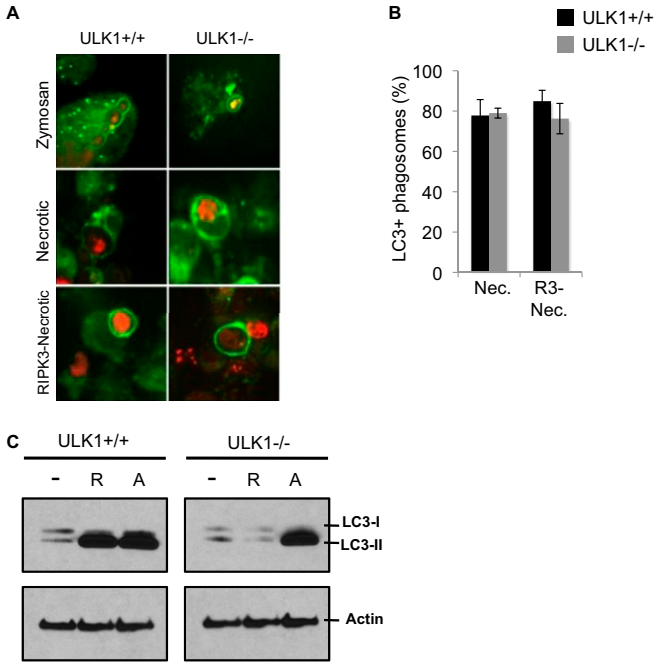

Fig. S6. ULK1 does not mediate LAP. (*A*) ULK1$^{+/+}$ GFP-LC3^{+} and ULK1$^{-/-}$ GFP-LC3^{+} macrophages were fed Alexa Fluor-594 zymosan (red), necrotic, or RIPK3-necrotic cells (SytoRed), and translocation of GFP-LC3 to the phagosome was followed by time-lapse video microscopy for 3 h (representative frames are shown; $n = 4$). (*B*) The percentage of GFP-LC3^{+} dead cell-containing phagosomes ($n > 100$/group) was obtained from three independent time-lapse videos (3 h each) of ULK1$^{+/+}$ and ULK1$^{-/-}$ macrophages. Error bars represent SD of four independent experiments. (*C*) ULK1$^{+/+}$ and ULK1$^{-/-}$ macrophages were fed with apoptotic cells ("A") for 2 h. Untreated (–) and rapamycin (R)-treated macrophages were obtained at 24 h. Cell lysates were prepared, and the levels of LC3-I and the active conjugated form LC3-II were determined by Western blot. As a loading control, the same membrane was restained for actin.

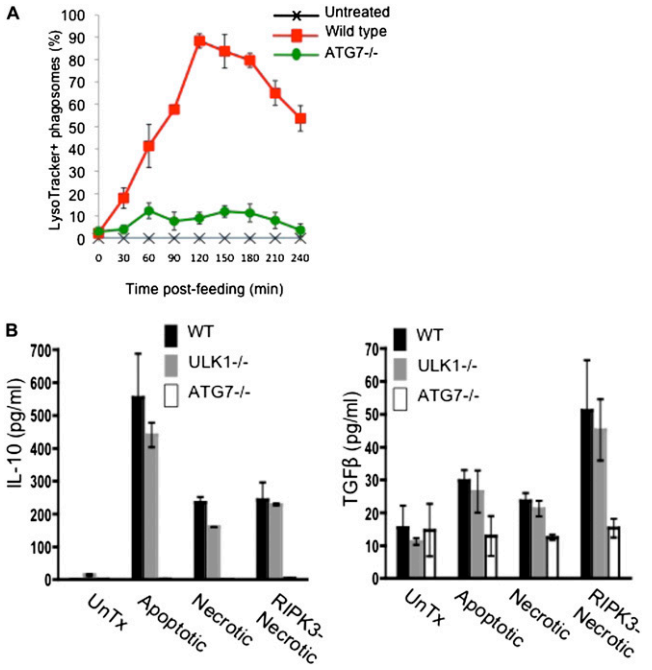

Fig. S7. The absence of LAP results in decreased production of anti-inflammatory cytokines in response to dead cell uptake. (*A*) LysM-Cre^{-} ATG7$^{f/f}$ GFP-LC3^{+} (ATG7$^{+/+}$) and LysM-Cre^{+} ATG7$^{f/f}$ GFP-LC3^{+} (ATG7$^{-/-}$) macrophages were preloaded with LysoTracker Red and fed with mCerulean-Spectrin MEFs induced to undergo apoptosis. Internalization, GFP-LC3 translocation, and phagosomal maturation were followed at 3-min intervals. Single cells were followed for at least 18 h ($n > 30$/group). Time course of phagosomal maturation as measured by the appearance of LysoTracker Red^{+} phagosomes is shown. Error bars represent SD of five independent experiments. (*B*) WT, LysM-Cre^{+} ATG7$^{f/f}$ (ATG7$^{-/-}$), and ULK1$^{-/-}$ macrophages were fed with apoptotic cells, necrotic cells, or RIPK3-necrotic cells, and supernatant cultures were analyzed at 24 h by Luminex technology for IL-10 (*Left*) and TGFβ (*Right*). Error bars represent SD of three independent experiments.

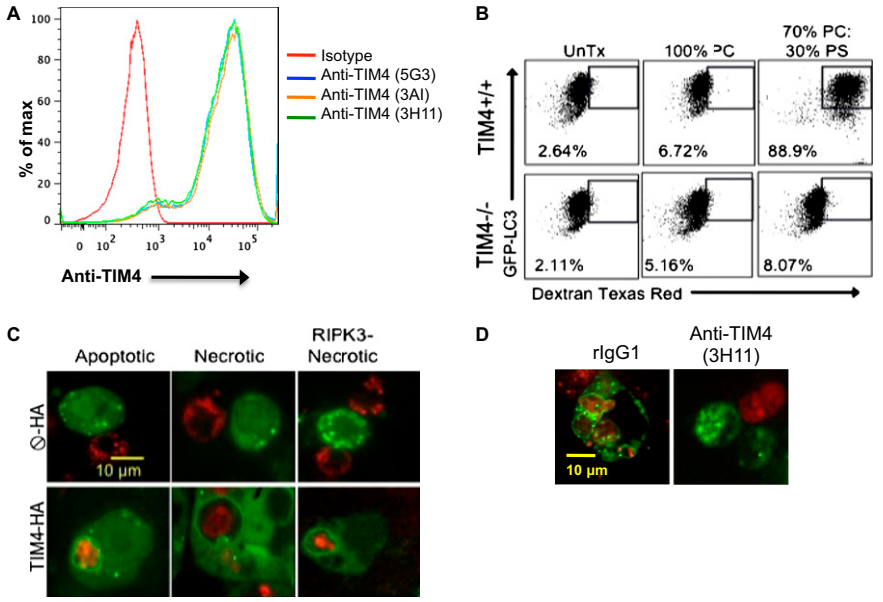

Fig. S8. TIM4 mediates LAP in response to engulfment of dead cells. (*A*) Bone marrow-derived macrophages were stained for F4/80 and TIM4 (clones 3A1, 5G3, and 3H11) and analyzed by flow cytometry. Histogram shows TIM4 expression of F480^{+} cells. (*B*) TIM4$^{+/+}$ GFP-LC3^{+} (*Upper panels*) and TIM4$^{-/-}$ GFP-LC3^{+} (*Lower panels*) macrophages were fed with 100% PC liposomes or 70% PC:30% PS liposomes (Texas Red). Internalization and colocalization of liposome fluorescence (Texas Red) with GFP-LC3 was analyzed by flow cytometry (n = 2). (*C*) RAW-GFP-LC3^{+} cells were transfected with empty vector (Ø-HA, *Upper panels*) or TIM4-HA (*Lower panels*) constructs. At 24 h after transfection, cells were fed apoptotic, necrotic, or RIPK3-necrotic cells (SytoRed), and phagocytosis and translocation of GFP-LC3 to the dead cell-containing phagosome was followed by time-lapse video microscopy for 3 h (representative frames are shown; n = 2). (*D*) GFP-LC3^{+} macrophages were treated with 25 μg/mL anti-TIM4 3H11 mAb or rIgG1 isotype control for 1 h. After this 1-h incubation, apoptotic cells (SytoRed) were added to the macrophage culture, and internalization and subsequent translocation of GFP–LC3 to the dead cell-containing phagosome was followed by time-lapse video microscopy for 3 h (representative frames are shown; n = 2).

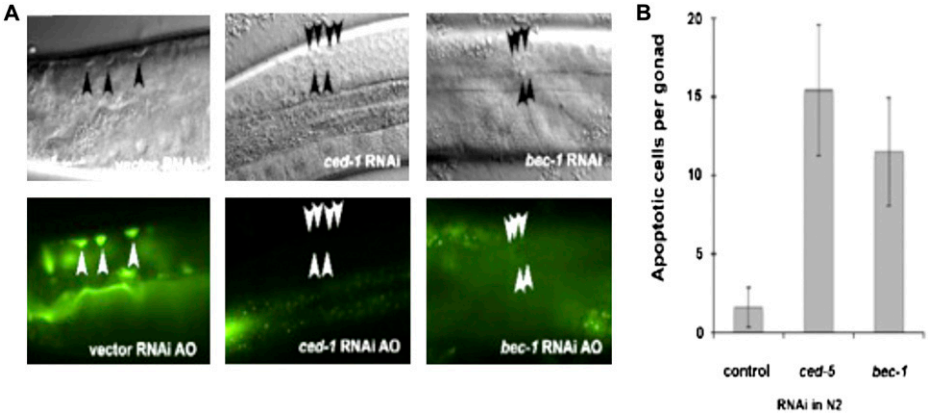

Fig. S9. Bec-1 is required for efficient cell-corpse clearance in the adult *Caenorhabditis elegans* hermaphrodite germ line. (*A*) N2 wild-type worms were treated with RNAi as indicated. DIC micrographs (*Upper panels*) or epifluorescence pictures (*Lower panels*) of *C. elegans* germ lines. Dorsal is at the top, and the germ line bend is to the right. Arrowheads indicate apoptotic germ cells or acridine orange (AO) staining of apoptotic corpses. (*B*) Quantification of germ-cell corpses in the corresponding genetic backgrounds. Animals were scored 24 h post L4/adult molt under DIC and epifluorescence. Error bars represent SD of $n >$ 12 animals.